FORM PTO-1390 (Modified) (REV 11-98) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: **MBHB00-210**

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR): **09/508570**

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP97/05378	**15 September 1997**	**15 September 1997**

TITLE OF INVENTION: **MULTIVALENT VACCINES**

APPLICANT(S) FOR DO/EO/US: **Francois Arminjon; Jean-Rene Cartier; Sandrine Lentsch-Graf; Laurent Marchal**

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ A copy of the International Search Report (PCT/ISA/210).
8. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☒ Certificate of Mailing by Express Mail
20. ☒ Other items or information:

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514 Rec'd PCT/PTO 13 MAR 2000

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/508570	PCT/EP97/05378	MBHB00-210

21. The following fees are submitted:.

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

		CALCULATIONS PTO USE ONLY
☒	Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2) paid to USPTO and International Search Report not prepared by the EPO or JPO	**$970.00**
☐	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but Internation Search Report prepared by the EPO or JPO	**$840.00**
☐	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO	**$690.00**
☐	International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4)	**$670.00**
☐	International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4)	**$96.00**

ENTER APPROPRIATE BASIC FEE AMOUNT =	**$970.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	**$130.00**	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	18 - 20 =	0	x $18.00	**$0.00**	
Independent claims	1 - 3 =	0	x $78.00	**$0.00**	
Multiple Dependent Claims **(check if applicable)**.			☐	**$0.00**	
TOTAL OF ABOVE CALCULATIONS			=	**$1,100.00**	
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) **(check if applicable)**.			☐	**$0.00**	
SUBTOTAL			=	**$1,100.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)).			+	**$0.00**	
TOTAL NATIONAL FEE			=	**$1,100.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).**			☐	**$0.00**	
TOTAL FEES ENCLOSED			=	**$1,100.00**	
				Amount to be: refunded	$
				charged	$

☒ A check in the amount of **$1,100.00** to cover the above fees is enclosed.

☐ Please charge my Deposit Account No. in the amount of to cover the above fees.
A duplicate copy of this sheet is enclosed.

☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **13-2490** A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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SIGNATURE

Michael S. Greenfield
NAME

37,142
REGISTRATION NUMBER

March 13, 2000
DATE

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
(Case No. MBHB00-210)

In the Application of:)
)
Arminjon *et al.*)
) **Examiner: J. Riley**
Serial No.: U.S. National Phase of)
PCT/EP97/05378)
) **Group Art Unit: 1807**
International Filing Date: September 15, 1997)
)
For: Multivalent Vaccines)

PRELIMINARY AMENDMENT

Asst. Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

Please consider the following amendments and remarks before examination on the merits.

AMENDMENTS

In the claims:

Please cancel claims 1-20.

Please add new claims 21-38:

21. A method for preparing a multi-component vaccine comprising at least:

a) pertussis toxoid and filamentous hemagglutinin in purified form,

b) tetanus toxoid,

c) diphtheria toxoid,

d) inactivated polio virus, and

e) a conjugate of a carrier molecule selected from tetanus toxoid and diphtheria toxoid and a capsular polysaccharide of *Haemophilus influenzae* type B,

wherein tetanus toxoid and diphtheria toxoid are adsorbed onto an aluminum salt before being mixed with the other components.

22. The method according to claim 21, wherein pertussis toxoid and filamentous hemagglutinin in purified form are adsorbed onto an aluminum salt before being mixed with the other components.

23. The method according to claim 21, wherein inactivated polio virus is mixed with the other components without being adsorbed onto an aluminum salt.

24. The method according to claim 21, wherein the aluminum salt is selected from a group consisting of aluminum hydroxide and aluminum phosphate.

25. The method according to claim 21, further comprising adding hepatitis B surface antigen adsorbed onto an aluminum salt.

26. The method according to claim 23, wherein mixing is conducted in the following order:

a) adsorbing tetanus toxoid and diphtheria onto an aluminum salt,

b) adsorbing pertussis toxoid and filamentous hemagglutinin in purified form onto an aluminum salt,

c) mixing the components obtained in a) with those obtained in b),

d) adding inactivated polio virus,

e) adding a solution of a conjugate of a carrier molecule selected from tetanus toxoid and diphtheria toxoid and a capsular polysaccharide of *Haemophilus influenzae* type B.

27. A method according to claim 25 wherein mixing is conducted in the following order:

a) adsorbing tetanus toxoid and diphtheria onto an aluminum salt,

b) adsorbing pertussis toxoid and filamentous hemagglutinin in purified form onto an aluminum salt,

c) mixing the components obtained in a) with those obtained in b),

d) adding inactivated poliovirus after c),

e) adding hepatitis B surface antigen previously adsorbed onto an aluminum salt after d),

f) adding a solution of a conjugate of a carrier molecule selected from tetanus toxoid and diphtheria toxoid and a capsular polysaccharide of *Haemophilus influenzae* type B after e).

28. The method according to claim 21, wherein said conjugate of a carrier molecule selected from tetanus toxoid and diphtheria toxoid and a capsular polysaccharide of *Haemophilus influenzae* type B is prepared in a phosphate buffer solution before being mixed with the other components.

29. The method according to claim 25, wherein hepatitis B surface antigen previously adsorbed onto aluminum salt is added separately from the other components within a dual chamber syringe.

30. A multi-component vaccine obtained by the method according to claim 21.

31. The multi-component vaccine according to claim 30, wherein the amounts of pertussis toxoid and filamentous hemagglutinin are between 5 and 30 μg in a single dose of said multi-component vaccine.

32. The multi-component vaccine according to claim 30, wherein the amounts of diphtheria toxoid and tetanus toxoid are between 5 and 30 LF in a single dose of said multi-component vaccine.

33. The multi-component vaccine according to claim 30 wherein the amounts of the different polioviruses are

a) between 20 and 50 D antigen units of poliovirus type1,

b) between 4 and 10 D antigen units of poliovirus type2, and

c) between 8 and 40 antigen units of poliovirus type3,

in a single dose of said multi-component vaccine.

34. A multi-component vaccine according to claim 10 wherein the composition of said vaccine comprises per 0.5 ml dose:

a) 25 μg pertussis toxoid;

b) 25 μg filamentous hemagglutinin;

c) 30 LF diphtheria toxoid;

d) 10 Lf tetanus toxoid;

e) 40 D antigen units poliovirus type 1;

f) 8 D antigen units poliovirus type 2;

g) 32 D antigen units poliovirus type 3;

h) 10 µg Haemophilus influenzae type B polysaccharide covalently bound to 20 µg tetanus toxoid;

i) 5 µg hepatitis B surface antigen;

j) 20 µMoles phosphates;

k) 5 µMoles carbonates;

l) 0.125 ml of 50 mM tris buffer; and

m) 0.306 mg aluminum salt.

35. The multi-component vaccine according to claim 30, wherein the composition of said vaccine comprises per 0.5 ml dose:

a) 25 µg pertussis toxoid;

b) 25 µg filamentous hemagglutinin;

c) 30 LF diphtheria toxoid;

d) 10 Lf tetanus toxoid;

e) 40 D antigen units poliovirus type 1;

f) 8 D antigen units poliovirus type 2;

g) 32 D antigen units poliovirus type 3;

h) 10 µg Haemophilus influenzae type B polysaccharide covalently bound to 20 µg tetanus toxoid;

i) 5 µg hepatitis B surface antigen;

j) 20 µMoles phosphates;

k) 5 µMoles carbonates;

l) 0.125 ml of 50 mM tris buffer; and

m) 0.356 mg aluminum salt.

36. A method for conferring protection in a host against disease caused by *Bordetella pertussis, Clostridium tetanii, Corynebacterium diphtheriae, Haemophilus influenzae, Poliovirus* and/or *Hepatitis B virus* using a multi-component vaccine according to claim 30.

37. A method of immunizing a human host against disease caused by infection by *Bordetella pertussis, Clostridium tetanii, Corynebacterium diphtheriae, Haemophilus influenzae, Poliovirus,* and/or *Hepatitis B virus*, which method comprises administering to the host a multi-component vaccine according to claim 30.

38. The method of claim 36 wherein the host is an infant.

REMARKS

The foregoing amendments are submitted to more clearly recite the claimed subject matter and do not introduce new subject matter.

If there are any questions or comments regarding this Response or application, the Examiner is encouraged to contact the undersigned attorney as indicated below.

Respectfully submitted,

Date: March 13, 2000

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TITLE OF INVENTION
MULTIVALENT VACCINES

BACKGROUND OF THE INVENTION

Infectious diseases remain a threat to human health despite decades of vaccine research. Combination vaccines which provide protection against multiple pathogens are very desirable to minimize the number of immunizations required to confer protection against multiple pathogens. The well documented phenomenon of antigenic competition complicates the development of multi-component vaccines. Antigenic competition refers to the observation that administering multiple antigens often results in a diminished response to certain antigens relative to the immune response observed when such antigens are administered individually.

The multiple pathogens against which the vaccines of the present invention provides protection are discussed in the context of the diseases they cause and the antigens from the pathogens which can be used in formulation of the present invention.

Whooping cough or pertussis is a severe, highly contagious upper respiratory tract infection caused by *Bordetella pertussis*. The World Health Organization estimates that there are 60 million cases of pertussis per year and 0.5 to 1 million associated deaths (ref. 1. Throughout this specification, various references are referred to in parenthesis to more fully describe the state of the art to which this invention pertains. Full bibliographic information for each citation is found at the end of the specification, immediately following the claims. The disclosures of these references are hereby incorporated by reference into the present disclosure). In unvaccinated populations, a pertussis incidence rate as high as 80% has been observed in children under 5 years old (ref. 2). Although pertussis is generally considered to be a childhood disease, there is increasing evidence of clinical and asymptomatic disease in adolescents and adults (refs. 3, 4, and 5).

The introduction of whole-cell vaccines composed of chemically- and heat-inactivated *B. pertussis* organisms in the 1940's was responsible for a dramatic reduction in the incidence of whooping cough caused by *B. pertussis*. The efficacy rates for whole-cell vaccines have been estimated at up to 95% depending on case definition (ref. 6). While infection with *B. pertussis* confers life-long immunity, there is increasing evidence for waning protection after immunization with whole-cell vaccines (ref. 3). Several reports citing a relationship between whole-cell pertussis vaccination, reactogenicity and serious side-effects led to a decline in

vaccine acceptance and consequent renewed epidemics (ref. 7). More recently, defined component pertussis vaccines have been developed.

Antigens for Defined Pertussis Vaccines

Various acellular pertussis vaccines have been developed and include the *Bordetella* pertussis antigens, Pertussis Toxin (PT), Filamentous haemagglutinin (FHA), the 69kDa outer membrane protein (pertactin) and fimbrial agglutinogens. PT and FHA are included in the formulations of the present invention and are described in more detail below.

Pertussis Toxin

Pertussis toxin is an exotoxin which is a member of the A/B family of bacterial toxins with ADP-ribosyltranserase activity (ref. 8). The A-moiety of these toxins exhibit the ADP-ribosyltransferase activity and the B-moiety mediates binding of the toxin to host cell receptors and the translocation of A to its site of action. PT also facilitates the adherence of *B. pertussis* to ciliated epithelial cells (ref. 9) and also plays a role in the invasion of macrophages by *B. pertussis* (ref. 10).

All acellular pertussis vaccines have included PT, which has been proposed as a major virulence factor and protective antigen (ref. 11, 12). Natural infection with B. pertussis generates both humoral and cell-mediated responses to PT (refs. 13 to 17). Infants have transplacentally-derived anti-PT antibodies (refs. 16, 18) and human colostrum containing anti-PT antibodies was effective in the passive protection of mice against aerosol infection (ref. 19). A cell-mediated immune (CMI) response to PT subunits has been demonstrated after immunization with an acellular vaccine (ref. 20) and a CMI response to PT was generated after whole-cell vaccination (ref. 13). Chemically-inactivated PT in whole-cell or component vaccines is protective in animal models and in humans (ref. 21). Furthermore, monoclonal antibodies specific for subunit S1 protect against *B. pertussis* infection (refs. 22 and 23).

The main pathophysiological effects of PT are due to its ADP-ribosyltransferase activity. PT catalyses the transfer of ADP-ribose from NAD to the G_i guanine nucleotide-binding protein, thus disrupting the cellular adenylate cyclase regulatory system (ref. 24). PT also prevents the migration of macrophages and lymphocytes to sites of inflammation and interferes with the neutrophil-mediated phagocytosis and killing of bacteria (ref. 25). A number of *in vitro* and *in vivo* assays have been used to asses the enzymatic activity of S1 and/or PT, including the ADP-ribosylation of bovine transducin (ref. 26), the Chinese hamster ovary (CHO) cell clustering assay (ref. 27), histamine sensitization (ref. 28), leukocytosis, and

NAD glycohydrolase. When exposed to PT, CHO cells develop a characteristic clustered morphology. This phenomenon is dependent upon the binding of PT, and subsequent translocation and ADP-ribosyltransferase activity of S1 and thus the CHO cell clustering assay is widely used to test the integrity and toxicity of PT holotoxins.

PT must be detoxified prior to its inclusion in a vaccine formulation. Several techniques of chemical detoxification have been described, including inactivation with formalin (ref. 46), glutaraldehyde (ref. 52), hydrogen peroxide (ref. 53) and tetranitromethane (ref. 54). Alternatively, mutant strains of *B. pertussis* or recombinant host cells expressing genetically detoxified PT may be used to prepare enzymatically inactive PT which retains immuological activity.

Filamentous Haemagglutinin

Filamentous haemagglutinin is a large (220 kDa) non-toxic polypeptide which mediates attachment of *B. pertussis* to ciliated cells of the upper respiratory tract during bacterial colonization (refs. 9, 29). Natural infection induces anti-FHA antibodies and cell mediated immunity (Refs. 13, 15, 17, 30 and 31). Anti-FHA antibodies are found in human colostrum and are also transmitted transplacentally (refs. 17, 18 and 19). Vaccination with whole-cell or acellular pertussis vaccines generates anti-FHA antibodies and acellular vaccines containing FHA also induce a CMI response to FHA (refs. 20, 32). FHA is a protective antigen in a mouse respiratory challenge model after active or passive immunization (refs. 33, 34). However, alone FHA does not protect in the mouse intracerebral challenge potency assay (ref. 28).

Acellular Pertussis Vaccines

The first acellular pertussis vaccine developed was the two-component PT + FHA vaccine (JNIH 6) of Sato et al. (ref. 46). This vaccine was prepared by co-purification of PT and FHA antigens from the culture supernatant of *B. pertussis* strain Tohama, followed by formalin toxoiding. Acellular vaccines from various manufacturers and of various compositions have been used successfully to immunize Japanese children against whopping cough since 1981 resulting in a dramatic decrease in incidence of disease (ref. 47). The JNIH 6 vaccine and mono-component PT toxoid vaccine (JNIH 7) were tested in a large clinical trial in Sweden in 1986. Initial results indicated lower efficacy than the reported efficacy of a whole-cell vaccine, but follow-up studies have shown it to be more effective against milder disease diagnosed by serological methods (refs. 48, 49, 50, 51). However, there was evidence

for reversion to toxicity of formalin-inactivated PT in these vaccines. These vaccines were also found to protect against disease rather than infection.

A number of new acellular pertussis vaccines are currently being assessed which include combinations of PT, FHA, and/or 69 kDa Outer Membrane Protein (Pertactin), and/or fimbrael agglutinogens.

Tetanus

Tetanus is an acute infection caused by *Clostridium tetani.* The disease is characterized by severe, painful muscle contractions, accompanied by hypersensitivity, hyperreflexia and increased autonomic stimulation of the affected body part(s). Mild stimuli may cause severe reflex muscle spasms. Fever due to extreme muscle spasm may be present. Tetanus may be generalized, involving the face, neck, abdomen and trunk or localized to a specific body part (injury site). Involvement of the masseter muscle of the face results in trismus or lockjaw giving rise to the classical facial expression known as "risus sardonicus" (ref. 78).

C. tetani exists as a nonpathogenic organism in the gut of humans and animals. The organism is also found in soil contaminated by feces and may survive in soil for years as infectious spores (ref. 79).

Tetanus results from the anaerobic growth of *C. tetani* and neurotoxin production in contaminated wounds. Infection is caused by the introduction of materials contaminated by organisms or spores into tissue. The most common scenario is infection through a penetrating injury. However, in many cases no history of injury is obtainable. The presence of necrotic or ischemic tissue facilitates the growth of the bacillus (ref. 78).

Prevention of infection is by vaccination and by good wound care including careful cleaning and debridement of devitalized tissues. Individuals with contaminated wounds and who have failed series should be given both tetanus vaccine and tetanus immune globulin.

Treatment of the syndrome is mainly supportive and may include respiratory support, administration of tetanus antitoxin and careful cleaning of infected wounds. Despite modern medical care the case fatality rates still run as high as 30 to 90% (ref. 79). This is particularly true tin the elderly. Natural infection does not always produce immunity from further infection.

Prevention of infection by vaccination is the most effective method of controlling the disease. Since the introduction of universal vaccination, tetanus has become extremely rare in developed countries. Cases occur almost exclusively in individuals who failed to complete their series of vaccinations or who have not received appropriate booster doses. Individuals should receive a booster dose once every ten years.

Diphtheria

Diphtheria is an acute infection caused by the bacteria *Corynebacterium diphtheriae*. The main site of infection is the upper respiratory tract (nose, pharynx, larynx and trachea) (ref. 80). The characteristic lesion, a result of the bacterial cytotoxin, are patches of greyish pseudomembrane surrounded by inflammation. This is accompanied by cervical lymphadenopthy, swelling and edema of the throat. In severe cases the swelling may progress to the point of obstruction (laryngeal diphtheria). Other complications include myocarditis, central nervous system effects (cranial, motor and sensory neuropathies such as ascending paralysis), and thrombocytopenia. Other mucosal membranes may be less frequently affected. The clinical presentation may vary from asymptomatic infection to fulminant multisystem, and death (ref. 79). Cutaneous and would infections with diphtheria are common in the tropics and have been frequently reported in the U.S. indigent population. The only reservoir for *C. diphtheriae* is man (ref. 79).

A presumptive diagnosis may be made on clinical observation of the characteristic lesions but should be confirmed by bacterial examination of the lesions. If there is a strong clinical suspicion of diphtheria, treatment should be initiated immediately with antibiotics (penicillin or erythromycin) and diphtheria antitoxin, even if the diagnosis is not confirmed. Mortality increases the longer one waits after the onset of clinical symptoms (ref. 80). The case fatality rate ranges from five to ten per cent despite modern medical care (ref. 79) and occurs mainly in the very young and in the elderly. Natural infection does not always produce immunity from further infection (ref. 80).

Transmission is by direct contact with secretions or discharges from an infected individual. Individuals are contagious as long as bacteria are observed in the secretions. This may last up to four weeks after infection. Transmission may also occur with infected fomites (ref. 79). Strict isolation of cases is recommended.

Rarely individuals may become carriers and shed organisms up to six months after infection. Unimmunized carriers should be promptly vaccinated with the full series. Treatment with antibiotics eliminates carriage and infectiousness of cases in 4 days (ref. 80).

Poliomyelitis

Both inactivated (IPV) and live attenuated (OPV) poliovirus vaccines have been effective in controlling poliomyelitis world wide. A combined DPT-IPV vaccine is currently licensed in Europe and in Canada and has been shown to be safe and effective in millions of children worldwide.

Haemophilus influenzae type b

Prior to the availability of effective vaccines, *Haemophilus influenzae* type b (Hib) was a major cause of meningitis invasive bloodborne infections in young children and was the main cause of meningitis in the first 2 years of life (ref. 81). Approximately 10% of *Haemophilus influenzae* meningitis victims die despite medical care. Permanent sequelae are common in survivors. Immunization against *Haemophilus influenzae* began in Canada in 1987 with a polysaccharide vaccine (polyribose ribitol phosphate [PRP]). Improved immunogenicity was achieved in children 18 months of age and older with the introduction in 1988 of a vaccine consisting of PRP conjugated to diphtheria toxoid (PRP-D). Since 1992, infant immunization has been possible with the licensure of PRP conjugate vaccines immunogenic in infants under 1 year of age (PRP conjugated with tetanus toxoid or PRP-T). Use of these *Haemophilus influenzae* conjugate vaccines has been associated with a dramatic decrease in the incidence of invasive *Haemophilus* infection in Canada and elsewhere (ref. 82). Two Canadian clinical studies involving nearly 900 children in British Columbia and Alberta demonstrated that lyophilized PRP-T may be reconstituted with DPT (COMBIPACK) (ref. 83) or with DPT-Polio Adsorbed (PENTA™) (ref. 84) in addition to the usual saline diluent. Clinical studies involving more than 100,000 children around the world have demonstrated the efficacy of lyophilized PRP-T (ActHib™). Over 90% achieve anti-PRP levels considered to be protective ($\geq$ 0.15 µg/ml) after 3 doses of PRP-T starting at 2 months or after a single does of PRP-T given after 12 months of age. The proportion achieving levels indicative of long term protection (>1.0 µg/ml) varies from 70 to 100% depending on the study. Millions of doses of PRP-T have been sold in the United States, Canada and Europe since 1992. Breakthrough cases of invasive haemophilus infection after vaccination with PRP-T are rare and may be associated with diseases such as immunodeficiency (ref. 85).

Combination Vaccines

Although there are many actual and potential benefits of vaccines that combine antigens to confer protection against multiple pathogens, these combinations may have a

detrimental effect on the immunogenicity of the individual components. Combinations of diphtheria and tetanus toxoids with whole cell pertussis vaccine (DTP) have been available for over 50 years and the antibody response to the combination is superior to that of the individual components, perhaps as a result of the adjuvant effect of the whole cell pertussis vaccine. DTP combinations that also include inactivated poliovirus vaccine are licensed in many jurisdictions, although the antibody response to the pertussis antigens may be diminished by this combination (refs 69 to 71). The effect of combining DTP vaccines with Hib conjugate vaccine have been variable. Studies with a French DTP and PRPT demonstrated similar safety but a decreased antibody response to PRP (ref. 72 to 73) whereas studies with a Canadian DTP and PRPT showed no effect on the PRP response but lower pertussis agglutinogens and increased injection site tenderness in the combined immunization group (refs 74,75).

Data are now becoming available on the effect of combining acellular pertussis/diphtheria/tetanus (APDT) vaccines with Hib conjugate vaccine. In two month old infants given three doses of an acellular pertussis-diphtheria-tetanus vaccine combined with a Hib conjugate vaccine, the antibody response to PRP was significantly lower than in the group given separate injections on the same day (ref. 76). Similar results were reported with another acellular pertussis-diphtheria-tetanus vaccine combined with PRPT given for the first three doses (ref. 77).

In contrast to other reported studies, children immunized with the combined vaccine had a superior antibody response to PRP, diphtheria, and several of the pertussis antigens when compared to children given PRP at a separate visit. A liquid combination of diphteria, tetanus, pertussis (DTP) and Haemophilus influenzae type b (PRP-T) was safe and at least as immunogenic as the lyophilized preparation which corresponds to the reconstitution of Hib with DTP (ref.86). There may be several reasons for the equivalent or better immunogenicity for these vaccines when given as a combined injection rather than the decreased immunogenicity reported with other products. All acellular pertussis vaccines are not identical in their antigenic content, method of toxoiding, adjuvant or preservative. However, increased immunogenicity has been reported with acellular pertussis vaccines containing PT, FHA, and 69K (ref. 77) and containing PT, FHA, 69K and fimbriae (ref. 76).

A five component APDT vaccine was found to have a protective efficacy of 85% (95% CI 81/89) in a phase III clinical trial recently completed in Sweden under the auspices of the National Institutes of Health (ref. 78).

Current commercially-available combination vaccines may not contain appropriate formulations of appropriate antigens in appropriate immunogenic forms to achieve a desired level of efficacy in a susceptible human population.

It would be desirable to provide efficacious combination vaccines comprising acellular pertussis components together with selected relative amounts of selected antigens such as tetatus toxoid, diphtheria toxoid, *Haemophilus influenzae* type B polysaccharide conjugate, poliovirus, and/or Hepatitis B Surface Ag. It would be highly desirable to develop a multivalent vaccine against diseases caused by infection by *Bordatella pertussis, Corynebacterium diphtheriae, Clostridium tetanae, Haemophilis influenzae,* poliovirus and hepatitis B virus. However, in order for such combination vaccines to be effective at achieving the criterion of seroprotection for each individual antigenic component, significant challenges in the form of antigenic competition and interference phenomena must be overcome.The present invention overcomes the limitations of the prior art and solves the problems of antigenic competition and interference by providing formulations of up to nine separate antigens designed to elicit seroprotection for up to six different infectious diseases.

SUMMARY OF THE INVENTION

The present invention is directed towards combination or multivalent vaccines containing a plurality of vaccine components suitable for the prevention, amelioration or treatment of multiple disease states which meet the criterion for seroprotection for each of said vaccine components, and methods of use thereof. In accordance with one aspect of the invention there is provided a multivalent vaccine that is able to prevent, ameliorate or treat up to six disease states in humans.

In a preferred embodiment of the present invention, there is provided a multivalent immunogenic composition for conferring protection in a host against disease caused by infection by *Bordetella pertussis, Corynebacterium diphtheriae, Clostridium tetanae,* poliovirus, hepatitis B virus and/or *Haemophilus influenzae*. The multivalent immunogenic composition of this embodiment of the present invention comprises pertussis toxoid and filamentous hemagluttinin, in purified form, tetanus toxoid, diphtheria toxoid, inactivated poliovirus, hepatitis B surface antigen, and a conjugate of a carrier molecule and a capsular polysaccharide of *Haemophilus influenzae* type b.

The immunogenic composition may be formulated as a vaccine for *in vivo* administration to the host wherein the individual components of the composition are formulated such that the immunogenicity of individual components is not impaired by other

individual components of the composition. In another embodiment, combinations of individual components may be provided as separate liquid suspensions and mixed prior to administration. For example, a dual-chamber bypass syringe can be provided with certain components present in the proximal chamber and remaining components present in the distal chamber of the syringe. In yet another embodiment, certain components of the immunogenic composition are provided in a lyophilized form suitable for reconstitution with a liquid mixture of other components of the immunogenic composition.

The immunogenic compositions may further comprise an adjuvant, particularly aluminum salts such as aluminum hydroxide or aluminum phosphate.

Such vaccine compositions may contain about 5 to about 30 ug of pertussis toxoid, about 5 to about 30 ug of filamentous hemagluttinin, about 5 to about 50 LF of diphtheria toxoid, about 5 to about 50 LF of tetanus toxoid, about 5 to about 20 ug of Hib conjugate and about 1 to about 10 ug HBsAg, all preferably in combination with IPV.

The inactivated poliovirus employed in the immunogenic composition of the invention generally comprises a mixture of inactivated poliovirus types 1,2 and 3. In one formulation, such mixtures of inactivated poliovirus types may comprise:

from about 20 to about 50 antigen units of poliovirus type 1;

from about 4 to about 10 antigen units of poliovirus type 2; and

from about 8 to about 40 antigen units of poliovirus type 3 in a single human dose.

The conjugate molecule may comprise a conjugate of suitable carrier protein, for example, tetanus toxoid or diphtheria toxoid, and polyribose ribitol phosphate (PRP) of Haemophilus influenzae type b. Such conjugate molecule may be provided in a lyophilized form, which is reconstituted for administration by combination with the other components. In a preferred formulation, the conjugate is employed in the form of about 10 ug of PRP conjugated to about 20 ug of tetanus toxoid.

In addition, the vaccine may also comprise an adjuvant, particularly aluminum hydroxide.

In another aspect of the invention, there is provided a method of immunizing a host against multiple diseases, comprising administering to the host, which may be human, an immunoeffective amount of the immunogenic composition or vaccine as provided herein.

Advantages of the present invention include a multivalent vaccine which can confer protection against a range of common diseases in a safe and efficaceous manner. The ability to provide a single vaccination against multiple diseases without interference between the immunogenic responses to the various immunogens is beneficial .

The use of the multivalent vaccine of the present invention will reduce the number of injections and vaccination visits necessary for immunization. This will be especially useful and advantageous for childhood immunization.

DETAILED DESCRIPTION OF THE INVENTION

Pertussis toxin (PT) (including genetically detoxified analogs thereof, as described in, for example, Klein *et al.*, U.S. Patent No. 5, 085, 862, incorporated herein by reference) may be produced by a variety of methods. For example, PT may be isolated from the culture supernatant of a B. pertussis strain using conventional methods such as that described by Sakura (ref. 55). PT is isolated by first absorbing culture supernatant onto a colun containing the dye-ligand gel matrix Affi-Gel Blue (Bio-Rad Laboratories, Richmond, CA). PT is eluted from this column by high salt, such as, 0.75 M magnesium chloride, and, after removing the salt, is passed through a column of fetuin-Sepharose affinity matrix composed of fetuin linked to cyanogen-bromide activated Sepharose. PT is eluted from the fetuin column using 4M magnesium salt.

Alternatively, the method of Irons *et al.* (ref 56) may be used. Culture supernatant is absorbed onto a CNBr-activatged Sepharose 4B colum to which haptogobin is first covalently bound. The PT binds to the adsorbent at pH 6.5 and is eluted from the column using 0.1M Tris/0.5M NaCl buffer by a stepwise change to pH 10.0.

Alternatively, the method described in U.S. Patent No. 4,705,686 granted to Scott *et al.* on November 10, 1987 and incorporated herein by reference thereto may be used. In this method culture supernatants or cellular extracts of *B. pertussis* are passed through a column of an anion exchange resin of sufficient capacity to adsorb endotoxin but permit *Bordetella* antigens to flow through or otherwise be separated from the endotoxin.

Alternatively, PT may be purified by using perlite chromatography, as described in EP Patent No. 336 736, incorporated herein by reference thereto.

Detoxification of PT

PT is detoxified to remove undesired activities which could cause side reactions of the final vaccine. Any of a variety of conventional chemical detoxification methods can be used, such as treatment with formaldehyde, hydrogen peroxide, tetranitro-methane, or glutaraldehyde.

For example, PT can be detoxified with glutaraldehyde using a modification of the procedure described in Munoz *et al.* (ref. 57). In this detoxification process purified PT is incubated in a solution containing 0.01 M phosphate buffered saline. The solution is made 0.05\ with glutaraldehyde and the mixture is incubated at room temperature for two hours, and then made 0.02 M with L-lysine. The mixture is further incubated for two hours at room temperature and then dialyzed for two days against 0.01 M PBS. In a particular embodiment, the detoxification process of EP Patent No. 336 736 may be used. Briefly, PT may be detoxified with glutaraldehyde as follows:

Purified PT in 75mM potassium phosphate at pH 8.0 containing 0.22M sodium chloride is diluted with an equal volume of glycerol to protein concentrations of approximately 50 to 400 ug/ml. The solution is heated to 37°C and detoxified by the addition of glutaraldehyde to a final concentration of 0.5% (w/v). The mixture is kept at 37°C for 4 hrs and then aspartic acid (1.5 M) is added to a final concentration of 0.25 M. The mixture is incubated at room temperature for 1 hour and then diafiltered with 10 volumes of 10 mM potassium phosphate at pH 8.0 containing 0.15M sodium chloride and 5t glycerol to reduce the glycerol and to remove the glutaraldehyde. The PT toxoid is sterile-filtered through a 0.2 uM membrane.

If recombinant techniques are used to prepare a PT mutant molecule which shows no or little toxicity, for use as the toxoided molecule, chemical detoxification is not necessary.

Purification of FHA

FHA may be purified from the culture supernatant essentially as described by Cowell *et al.* (ref. 58).. Growth promoters, such as methylated beta-cyclodextrins, may be used to increase the yield of FHA in culture supernatants. The culture supernatant is applied to a hydroxylapatite column. FHA is adsorbed onto the column, but PT is not. The column is extensively washed with Triton X-100 to remove endotoxin. FHA is then eluted using 0.5M NaCl in 0.1M sodium phosphate and, if needed, passed through a

fetuin-Sepharose column to remove residual PT. Additional purification can involve passage though a Sepharose CL-6B column.

Alternatively, FHA may be purified using monoclonal antibodies to the antigen, where the antibodies are affixed to a CNBr-activated affinity column (ref. 59).

Alternatively, FHA may be purified by using perlite chromatography as described in the above-mentioned EP 336 736.

PT + FHA Vaccines

Such a vaccine could be prepared as described in references 87 and 88.

Hib antigens

Such antigens can be based on the capsular polysaccharide (PRP) conjugated with a carrier protein. The polymer is a polymer of ribose, ribitol and phosphate. Tipically, the carrier protein is a diphteria or tetanus toxoid or an outer membran of N.meningitidis. Such conjugates are for example disclosed in EP 161,188, EP 208,375, EP 477,508, US 4,365,170 or US 4,673,574.

Such polysaccharide conjugates may be prepared by any known coupling technique as described for examples in WO 93/15760 or in patents cited in the previous sentence.

If one of the Hib antigens selected for the composition is a Capsular Polysaccharide Tetanus Toxoid Conjugate (PRP-T is an example) and if an aluminium salt (aluminium hydroxid is an example) is used in the composition, the Capsular Polysaccharide Tetanus Toxoid Conjugate is less stable and less immunogen than without aluminium salt. In such cases, these problems of stability and immunogenicity can be solved by using the technology described in PCT/FR96/00791. In brief, it consists in adding anions to the aluminium salts. Said anions can be phosphates, citrates. Phosphates can be provided by a monopotassium phosphate, dissodium phosphate solution. A combination of phosphates and carbonates (sodium carbonate, sodium bicarbonate) can be used as anions too.

Polio virus vaccine

The poliovirus component of the combination may be the Salk inactivated polio vaccine.

Selected Multivalent Vaccine Formulations

In selected embodiments, the invention provides vaccines with the following characteristics , all of which may be administered by intramuscular injection:

One formulation comprises a combination of component pertussis vaccine combined with diphtheria and tetanus toxoids, inactivated poliovirus, Haemophilus influenza type B polysaccharide conjugate, and Hepatitis B Surface Ag and is termed Full liquid DTacP-IPV-PRP~T-HBsAg.

Each 0.5 ml human dose of Full liquid DTacP-IPV-PRP~T-HBsAg was formulated to contain about:

25 µg Pertussis toxoid (PT)
25 µg Filamentous haemagglutinin (FHA)
30 LF Diphtheria toxoid
10 LF Tetanus toxoid
40 D antigen units Poliovirus type 1
8 D antigen units Poliovirus type 2
32 D antigen units Poliovirus type 3
10 µg Haemophilus influenza type B polysaccharide covalently bound to
20 µg Tetanus protein
5 µg Hepatitis B Surface Ag
20 µMoles phosphates
5 µMoles carbonates
0.125 ml tris 50mMolaire buffer comprising saccharose in 42,5 %
0.306 mg Aluminum salts

Another formulation, which is referred to herein as "DTaP-IPV-PRP~T/HBsAg" or the "dual chamber format", is comprised of a mixture of antigens in solution in the proximal chamber of a bypass syringe; the remaining components are provided in the distal chamber of the bypass syringe. The resulting composition is the same as that provided hereinabove with the difference that aluminium salts are present in a quantity of 0,356 mg.

Vaccine Preparation and Use

Immunogenic compositions, suitable to be used as vaccines, may be prepared from the immunogens as disclosed herein. The vaccine elicits an immune response in a subject which produces antibodies.

Immunogenic compositions including vaccines may be prepared as injectibles, as liquid solutions or emulsions. The immunogens may be mixed with pharmaceutically acceptable excipients which are compatible with the immunogens. Such excipients may include water, saline, dextrose, glycerol, ethanol, and combinations thereof. The immunogenic compositions and vaccines may further contain auxiliary substances, 20 such as wetting or emulsifying agents, pH buffering agents, or adjuvants to enhance the effectiveness thereof.

If difficulties concerning the stability of the Hib antigen(s) as PRP-T appeared, these difficulties could be solved by using the teachings of reference 89.

Immunogenic compositions and vaccines may be administered parenterally, by injection subcutaneously or intramuscularly. The immunogenic preparations and vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective, immunogenic and protective. The quantity to be administered depends on the subject to be treated, including, for example, the capacity of the immune system of the individual to synthesize antibodies, and, if needed, to produce a cell-mediated immune response.

Suitable regimes for initial administration and booster doses are also variable, but may include an initial administration followed by subsequent administrations. The dosage may also depend on the route of administration and will vary according to the size of the host. Immunogenicity can be significantly improved if the antigens are coadministered with adjuvants, commonly used as 0.005 to 0.5 percent solution. Adjuvants enhance the immunogenicity of an antigen but are not necessarily immunogenic themselves.

Adjuvants may act by retaining the antigen locally near the site of administration to produce a depot effect facilitating a slow, sustained release of antigen to cells of the immune system. Adjuvants can also attract cells of the immune system to an antigen depot and stimulate such cells to elicit immune responses.

Immunostimulatory agents or adjuvants have been used for many years to improve the host immune responses to, for example, vaccines. Intrinsic adjuvants, such as lipopolysaccharides, normally are the components of the killed or attenuated bacteria used as vaccines. Extrinsic adjuvants are immunomodulators which are typically noncovalently linked to antigens and are formulated to enhance the host immune responses. Thus, adjuvants have been identified that enhance the immune response to antigens delivered parenterally.

Some of these adjuvants are toxic, however, and can cause undesirable side effects, making them unsuitable for use in humans and many animals. Indeed, only aluminum hydroxide and aluminum phosphate (collectively commonly referred to as alum) are routinely used as adjuvants in human and veterinary vaccines. The efficacy of alum in increasing antibody responses to diphtheria and tetanus toxoids is well established.

A wide range of extrinsic adjuvants can provoke potent immune responses to antigens. These include saponins complexed to membrane protein antigens (immune stimulating complexes), pluronic polymers with mineral oil, killed mycobacteria in mineral oil, Freund's complete adjuvant, bacterial products, such as muramyl dipeptide (MDP) and lipopolysaccharide (LPS), as well as 10 lipid A, and liposomes.

To efficiently induce humoral immune responses (HIR) and cell-mediated immunity (CMI), immunogens are often emulsified in adjuvants. Many adjuvants are toxic, inducing granulomas, acute and chronic inflammations (Freund's complete adjuvant, FCA), cytolysis (saponins and Pluronic polymers) and pyrogenicity, arthritis and anterior uveitis (LPS and MDP). Although FCA is an excellent adjuvant and widely used in research, it is not licensed for use in human or veterinary vaccines because 20 of its toxicity.

Desirable characteristics of ideal adjuvants include:

(1) lack of toxicity;
(2) ability to stimulate a long-lasting immune response;
(3) simplicity of manufacture and stability in long-term storage;
(4) ability to elicit both CMI and HIR to antigens administered by various routes;
(5) synergy with other adjuvants;
(6) capability of selectively interacting with populations of antigen presenting cells (APC):
(7) ability to specifically elicit appropriate TH1 or TH2 cell-specific immune responses; and
(8) ability to selectively increase appropriate antibody isotype levels (for example, IgA) against antigens.

U.S. Patent No. 4,855,283, granted to Lockhoff *et al.* on August 8, 1989 which is incorporated herein by reference thereto, teaches glycolipid analogues including N-glycosylamides, N-glycosylureas and N-glycosylcarbamates, each of which is substituted in the sugar residue by an amino acid, as immuno-modulators or adjuvants. Thus, Lockhoff *et al.* (U.S. Patent No. 4,855,283 and ref. 60) reported that N-glycolipid analogs displaying structural similarities to the naturally occurring glycolipids, such as glycosphingolipids and glycoglycerolipids, are capable of eliciting strong immune responses in both herpes simplex virus vaccine and pseudorabies virus vaccine. Some glycolipids have been synthesized from

long chain alkylamines and fatty acids that are linked directly with the sugars through the anomeric carbon atom, to mimic the functions of the naturally occurring lipid residues.

U.S. Patent No. 4,258,029 granted to Moloney and incorporated herein by reference thereto, teaches that octadecyl tyrosine hydrochloride (OTH) functions as an adjuvant when complexed with tetanus toxoid and formalin inactivated type I, II and III poliomyelitis virus vaccine. Also, Nixon-George *et al.* (ref. 61), reported that octodecyl esters of aromatic amino acids complexed with a recombinant hepatitis B surface antigen, enhanced the host immune responses against hepatitis B virus.

EXAMPLES

Examples are provided solely for purposes of illustration and are not intended to limit the scope of the invention. Changes in form and substitution of equivalents are contemplated as circumstances may suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitation.

Methods of protein biochemistry, fermentation and immunology used but not explicitly described in this disclosure and these Examples are amply reported in the scientific literature and are well within the ability of those skilled in the art.

Example 1:

Preparation of Full liquid formulation DTacP-IPV-PRP~T-HBsAg

A preferred formulation of a vaccine composition of the present invention comprises a liquid suspension of an immunoeffective amount of up to nine separate antigens, selected to elicit protection against as many as six infectious agents. This formulation, in which all of said antigens are present in solution for convenient administration to a human host, is designated or "Full liquid DtacP-IPV-PRP~T-HBs" or "full liquid" for short. The preferred method of manufacture of the full liquid formulation of the present invention is as follows.

An acidified aluminium hydroxide gel suspension is prepared by mixing at room temperature with pharmaceutical grade water. Successive additions of diphtheria toxoid (DT) and tetanus toxoid (TT) are then made to the gel suspension. The order of addition of these components is not critical, but the solution is preferably stirred for at least 30 minutes then allowed to settle for at least 30 minutes after the addition of each individual antigen

component. Pertussis toxoid (PT) and filamentous haemagluttinin (FHA) are each adsorbed separately to aluminum salts and concentrated if necessary. These components are added to the mixture above, stirred for at least 30 minutes, and allowed to settle overnight.

At this point, carbonate buffers in Medium 199 are added, preferably through a 0.2μm filter, and either NaOH (2.5M) or acetic acid (10%) is added as needed to adjust the pH to about 7 to 7.2.

Next, IPV is introduced to an appropriate concentration with pharmaceutical grade water and introduced into the mixture, preferably through a 0.2 μm filter. The pH is then adjusted to a value ranging from 6.8 to 7.

Next, HBsAg, which has been previously adsorbed to aluminium salts, is added and stirred for at least 30 minutes.

Phosphate buffer, Tris-sucrose buffer and Water For Injection are added on *Haemophilus influenzae* type b polysaccharide conjugate (Hib) concentrate solution.

Finally, the buffered Hib solution is added to the other vaccine components, preferably to a 0.22μm filter, and stirred at least 30 minutes.

The resulting suspension obtained generally from the process set forth above is referred to as the full liquid bulk product. This bulk is then used to prepare the individual 0.5ml doses for use in clinical studies and vaccination procedures. Those skilled in the art will appreciate that the orders of addition of individual components, buffers used to dilute individual components, methods of addition and mixing, acids and bases used to adjust pH and mixing conditions can be modified without deviating from the spirit of the invention claimed herein.

Example 2:

Preparation of dual chamber formulation DtacP-IPV-PRP~T/HBsAg

Another formulation of a vaccine composition of the present invention comprises a liquid suspension of an immunoeffective amount of up to eight separate antigens, selected to elicit protection against as many as five infectious agents is present in a first, proximal chamber of a 1mL by-pass syringe and an immunoeffective amount of another antigen, selected to elicit protection against an additional infectious agent, is present in a second, distal chamber of said by-pass syringe. This formulation, in which all antigens are present in

solution and in which certain of said antigens are disposed within the distal chamber of a bypass syringe and remaining antigen(s) are disposed within the proximal chamber of a bypass syringe is designated "Dual Chamber DtacP-IPV-PRP~T-HBs" or "dual chamber" for short.

One method of manufacture of the dual chamber formulation of the present invention entails a minor modification of the manufacturing method provided for the full liquid bulk in Example 1. All of the steps provided for manufacture of the full liquid bulk are the same, except that the step in which HBsAg is added to the mixture is omitted. The resulting solution is designated the DtacP-IPV-PRP~T bulk. 0.5mL of the DtacP-IPV-PRP~T bulk is disposed within the distal chamber of a 1.0 cc by-pass syringe. 0.5ml of an immunoeffective dose of HBsAg which has been previously adsorbed to aluminium salts is disposed within the proximal chamber of said by-pass syringe.

Example 3:

Clinical Trials

Clinical trials were performed in humans as described herein to establish the safety, nonreactogenicity and utility of the multivalent vaccine compositions of the present invention. In particular, adverse reactions were recorded (as show, for example, in Tables 1 and 2 below) and immune responses to each of the antigens contained in the vaccines (as shown, for example, in Tables 3-5 below) were determined. The full liquid and dual chamber formulations were each analyzed in a open, non comparative, randomized study.

350 infants were recruited in a two-arm randomized study to receive a total of four (4) inections of vaccine. The infants were divided into two equal groups. The first group (Group 1) received the multivalent vaccine in the full liquid format. The second group (Group 2) received the multivalent vaccine in a dual chamber format. (As described herein, the "dual chamber" formulation involves the use of an on aluminium salt adsorbed Hepatitis B surface antigen (HBsAg) in the proximal chamber of the syringe, with the remaining components of the vaccine present in a buffered solution in the distal chamber of the syringe).

The vaccination schedule was comprised of an intramuscular injection of the given at two (2), three (3) and four (4) months of age and a final injection at some point between 12 and 14 months of age. Vaccines were administered by intramuscular injection, perpendicular to the skin surface, into the anterolateral aspect of the thigh. Blood samples were taken for antibody titration immediately prior to vaccination and one month after dose 3.

Adverse events were monitored for one month after each immunization and local reactions at the site of injection were recorded within three (3) days after each injection. No vaccine related serious adverse event was reported during the entire study period.

Local reactions were few and transient and the vaccines were well tolerated.

The IgG response to each component of the multivalent vaccines were compared by standard serological analysis in which the antibody titers following dose 3 were compared to pre-immunization titers. For the most part, there was no significant difference in the results obtained by immunization with the full liquid formulation compared to the dual chamber formulation. Both vaccines provide an excellent and seroprotective immune response against each antigen of their composition. These results are shown in their entirety, on a component by component basis, in Tables 3(a)-(e) below and final results, in terms of seroprotection, are provided in Table 4 below.

In the table 3, the following conventional abbreviations are used. *n* = the number of subject evaluated. GMT = geometric mean titer, and CI = the confidence interval around each GMT value, as determined by standard statistical methodology.

In table 4, the criteria for seroprotection correspond to the reference commonly admitted by the vaccinology community for each component in terms of expected antibody response obtained after a primary immunisation consisting of three doses given 1 to 2 months apart or after a booster immunisation given about one year after the first immunisation. The criterion for seroprotection for the PT and FHA antigens are 4-fold rise between the pre and post primary series titers and post fourth dose titers. SPR is the seroprotection rate and corresponds to the percentage of subjects fulfilling the criterion of response . GMT has the same meaning as in table 3.

Table 3(a) HBsAg antibody response by vaccine group

VACCINE GROUP	Full Liquid DtaP-IPV-PRP~T - HBsAg		Dual Chamber DTaP-IPV-PRP~T/HBsAg	
	Pre-immunization	Post-dose 3	Pre-immunization	Post-dose 3
n	*108*	*107*	*111*	*112*
GMT	4.88	142	5.68	72.70
[95 % CI]	[2.8-8.7]	[102-197]	[3.2-10.1]	[53.5-99]

Table 3(b) PT and FHA (EIA) antibody responses by vaccine group

VACCINE GROUP	Full Liquid DtaP-IPV-PRP~T - HBsAg		Dual Chamber DTaP-IPV-PRP~T/HBsAg	
	Pre-immunization	Post-dose 3	Pre-immunization	Post -dose 3
PT (EIA - EUImL)				
n	*103*	*108*	*107*	*112*
GMT	2.39	53.30	2.56	61.30
[95 % CI]	[1.9-3.0]	[48.0-59.1]	[2.0-3.2]	[55.6-67.6]
FHA (EIA - EUImL)				
n	102	107	108	112
GMT	4.73	97.70	4.88	133.0
[95 % CI]	[3.7-6.0]	[86.1-111]	[4.0-6.0]	[119-149]

Table 3 (c) Tetanus and diphtheria antibody responses

VACCINE GROUP	Full Liquid DTaP-IPV-PRP~T -HBsAg		Dual Chamber DTaP-IPV-PRP~T/ HBsAg	
	Pre-immunization	Post-dose 3	Pre-immunization	Post-dose 3
Tetanus (EIA - IU/mL)				
n	*100*	*105*	*101*	*111*
GMT	0.42	0.73	0.45	0.90
[95 % CI]	[0.31-0.56]	[0.62-0.90]	[0.34-0.60]	[0.75-1.10]
Diphtheria (EIA - IU/mL)				
n	*95*	*105*	*95*	*107*
GMT	0.09	0.19	0.11	0.14
[95 % CI]	[0.06-0.14]	[0.15-0.24]	[0.07-0.16]	[0.11-0.18]

Table 3(d) Type 1, 2 and 3 poliomyelitis virus neutralising antibody response

VACCINE GROUP	Full Liquid DtaP-IPV-PRP~T-HBsAg		Dual Chamber DTaP-IPV-PRP~T/ HBsAg	
	Pre-immunization	Post dose 3	Pre-immunization	Post dose 3
Polio Type 1 (1/dil.)				
n	*107*	*108*	*110*	*112*
GMT	63.8	254	47.4	305
[95 % CI]	[46.5-87.5]	[196-330]	[35.3-63.6]	[232-403]
Polio Type 2 (1/dil.)				
n	*107*	*108*	*110*	*112*
GMT	85.3	115	67	125
[95 % CI]	[65.6-111]	[87.6-151]	[53.2-84.4]	[93.4-166]
Polio Type 3 (1/dil.)				
n	*107*	*108*	*110*	*112*
GMT	46.9	290	37.9	333
[95 % CI]	[35.6-61.8]	[216-390]	[28.5-50.5]	[252-439]

Table 3 (e) PRP antibody response

VACCINE GROUP	Full Liquid DtaP-IPV-PRP~T - HBsAg		Dual Chamber DTaP-IPV-PRP~T/HBsAg	
	Pre-immunization	Post-dose 3	Pre-immunization	Post-dose 3
PRP (RIA - µg/mL)				
n	*108*	*108*	*110*	*112*
GMT	0.08	1.46	0.09	3.05
[95 % CI]	[0.07-0.1]	[1.1-1.9]	[0.08-0.12]	[2.3-4.0]

Table 4. Seroprotection for the full Liquid Formulation

Antigen	Criteria for seroprotection	n	% of subjects protected
PRP	% ≥0.15 µg/ml	152	92.1
HBsAg	% >10 mIU/ml	151	92.7
DT	% >0.01 IU/ml	144	99.3
TT	% >0.01 IU/ml	147	100
Polio type 1	% titer >5 (Neut.)	152	100
Polio type 2	% titer >5 (Neut.)	152	100
Polio type 3	% titer >5 (Neut.)	152	100

PT (EU/mL)	% titer ≥ 4-fold increase	146	87.0
FHA (EU/mL)	% titer ≥ 4-fold increase	145	87.6

The safety and immunogenicity results obtained one month after the 2,3,4 months primary series demonstrate the utility of the multivalent vaccines of the present invention. For D, T, and IPV antigens, the immune response was excellent. For the acellular pertussis components, more than eighty-seven percent of these infants showed a four-fold-rise in terms of anti PT anti FHA antibodies.

A good immune response was demonstrated for PRP at the 0.15 µg level, as well as for Hepatitis B, with 92% of seroprotection for both of them. These results are shown in Tables 3 and 4 above.

Results of Booster Vaccination.

The IgG response to each component of the multivalent vaccines tested were compared by standard serological analysis in which the antibody titers following the final, booster dose given at 12-14 months were compared to pre-booster (post dose 3) titers. These results are shown in their entirety, on a component by component basis, in Tables 5(a)-(e) below and final results, in terms of seroprotection, are provided in Table 6 below. All abbreviations in the following tables are as defined previously for Tables 3(a)-(e) and 4 above.

Table 5 (a) HBsAg antibody booster response

VACCINE GROUP	Full Liquid DtaP-IPV-PRP~T - HBsAg		Dual Chamber DtaP-IPV-PRP~T/HBsAg	
	Pre-Booster	Post-Booster	Pre-Booster	Post-Booster
HBsAg (RIA-AUSAB mIU/mL)				
n	*125*	*130*	*114*	*119*
GMT	50.4	1458	37.1	530
[95 % CI]	[38.1-66.8]	[1056-2013]	[29.1-47.3]	[399-704]

Table 5 (b) Pertussis antibody booster responses (PT and FHA)

VACCINE GROUP	Full Liquid DtaP-IPV-PRP~T - HBsAg		Dual Chamber DTaP-IPV-PRP~T/HBsAg	
	Pre-Booster	Post-Booster	Pre-Booster	Post-Booster
PT (EIA - EUImL)				
n	*124*	*129*	*112*	*115*
GMT	15.3	87.7	15	101
[95 % CI]	[13.5-17.4]	[79.9-96.2]	[13.1-17.2]	[90.6-112]
FHA (EIA - EUImL)				
n	*123*	*129*	*111*	*115*
GMT	28.9	149	38.6	168
[95 % CI]	[25.6-32.6]	[132-168]	[34.4-43.4]	[152-187]

Table 5 (c) Tetanus and diphtheria antibody booster responses

VACCINE GROUP	Full Liquid DtaP-IPV-PRP~T -HBsAg		Dual Chamber DTaP-IPV-PRP~T/ HBsAg	
	Pre-Booster	Post-Booster	Pre-Booster	Post-Booster
Tetanus (EIA - IU/mL)				
n	*115*	*129*	*105*	*115*
GMT	0.31	7.14	0.39	7.51
[95 % CI]	[0.3-0.4]	[6.2-8.1]	[0.3-0.5]	[6.5-8.6]
Diphtheria (EIA - IU/mL)				
n	*103*	*128*	*102*	*115*
GMT	0.04	1.17	0.04	0.98
[95 % CI]	[0.03-0.05]	[0.9-1.4]	[0.03-0.05]	[0.8-1.2]

Table 5 (d) Type 1, 2 and 3 poliomyelitis virus antibody booster response

VACCINE GROUP	Full Liquid DtaP-IPV-PRP~T-HBsAg		Dual Chamber DTaP-IPV-PRP~T/ HBsAg	
	Pre-Booster	Post-Booster	Pre-Booster	Post-Booster
Polio Type 1 (1/dil.)				
n	*120*	*124*	*104*	*111*
GMT	45.8	3113	47.3	2576
[95 % CI]	[35.2-59.6]	[2616-3705]	[34.6-64.7]	[2027-3274]
Polio Type 2 (1/dil.)				
n	*121*	*125*	*107*	*113*
GMT	32.8	2496	29.2	2237
[95 % CI]	[25-43]	[2057-3029]	[21-40.6	[1805-2772]
Polio Type 3 (1/dil.)				
n	*121*	*127*	*109*	*113*
GMT	50.2	3938	43.6	3101
[95 % CI]	[37.9-66.4]	[3327-4663]	[31.5-60.4]	[2434-3951]

Table 5 (e) PRP antibody booster response

VACCINE GROUP	Full Liquid DTaP-IPV-PRP~T - HBsAg		Dual Chamber DTaP-IPV-PRP~T/HBsAg	
	Pre-Booster	Post-Booster	Pre-Booster	Post-Booster
PRP (RIA - µg/mL)				
n	*125*	*129*	*112*	*119*
GMT	0.6	28.6	0.8	31.3
[95 % CI]	[0.3-0.6]	[22.3-36.7]	[0.6-1.0]	[24.1-40.8]

Table 6 seroprotection for the full Liquid Formulation

Components	Criterion for seroprotection	Post-dose 3		Post-dose 4	
		SPR(%)	GMT	SPR(%)	GMT
PRF	% 0.15 µg/mL	91.7	1.5	100.0	28.6
HBs	%[3] 10mIU/mL	91.6	142.0	98.5	1458.0
Diphteria	%[3]0.01 IU/mL	99.0	0.2	100.0	1.2
Tetanus	%[3]0.01 IU/mL	100.0	0.7	100.0	7.1
Polio 1	% titre [3] 5 (Neut)	100.0	254.0	100.0	3113.0
Polio 2	% titre [3] 5 (Neut)	100.0	115.0	100.0	2496.0
Polio 3	% titre [3] 5 (Neut)	100.0	290.0	100.0	3938.0
PT	% 4-fold rise (EU/mL)	88.3	53.3	69.4	87.7
FHA	% 4-fold rise (EU/mL)	87.7	97.7	69.2	149.0

For all antigens, we observed an excellent booster effect induced by the fourth dose confirming the presence of an excellent immune memory induced by this accelerated 2-3-4 month primary series.These results are shown in Tables 5 and 6 above.

The compositions of the invention provide a seroprotection against each disease.

SUMMARY OF THE DISCLOSURE

The preparation of numerous multivalent vaccines are described clearly above. Extensive clinical trials described above clearly demonstrate that the multivalent immunological compositions of the present invention are safe and efficacious for conferring protection against a broad range of pathogens.

These results are surprising insofar as mixtures of numerous vaccine components may have been expected to contribute to well-recognized phenomena of antigenic competition or interference, whereby certain vaccine components which would be capable of conferring seroprotection when introduced individually into an immunocompetent host become less effective when introduced in combination with other antigens. Thus, the vaccines of the present invention simplify the immunization process and greatly minimize the number of separate immunizations needed to protect pediatric patients from infection with *Bordatella*

pertussis, Corynebacterium diphtheriae, Clostridium tetanae, Haemophilus influenzae, poliovirus and Hepatitis b virus.

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CLAIMS

We claim:

1. A multi-valent immunogenic composition for conferring protection in a host against disease caused by *Bordetella pertussis, Clostridium tetani, Corynebacterium diphtheriae, Haemophilus influenzae,* poliovirus and/or Hepatitis B virus

2. A multi-valent immunogenic composition for conferring protection in a host against disease caused by *Bordetella pertussis, Clostridium tetani, Corynebacterium diphtheriae, Haemophilus influenzae,* poliovirus and/or Hepatitis B virus comprising:

   (a) pertussis toxoid and filamentous haemagluttinin in purified form,
   (b) tetanus toxoid,
   (c) diphtheria toxoid,
   (d) inactivated polio virus,
   (e) Hepatitis B surface Ag, and
   (f) a conjugate of a carrier molecule selected from tetanus toxoid and diphtheria toxoid and a capsular polysaccharide of *Haemophilus influenzae* type B.

3. The immunogenic composition of claim 2 formulated as a vaccine for *in vivo* administration to the host wherein the individual components of the composition are formulated such that the immunogenicity of individual components is not impaired by other individual components of the composition.

4. The immunogenic composition of claim 2 formulated as a vaccine for *in vivo* administration to the host, which confers an antibody titer superior to the criterion for seroprotection for each antigenic component for an acceptable percentage of human subjects.

5. The immunogenic composition of claim 3 further comprising an adjuvant.

6. The immunogenic composition of claim 5 wherein the adjuvant is aluminum salts .

7. The immunogenic composition of claim 3 wherein said pertussis toxoid is present in an amount of about 5 to about 30 ug and said filamentous hemagluttinin is present in an amount of about 5 to about 30 ug, in a single dose.

8. The immunogenic composition of claim 7 containing about 25 ug of pertussis toxoid and about 25 ug of filamentous haemagluttinin in a single human dose.

9. The vaccine of claim 7 wherein said diphtheria toxoid is present in an amount of about 5 to about 50 LF and said tetanus toxoid is present in an amount of about 5 to about 50 LF.

10. The vaccine of claim 9 wherein said diphtheria toxoid is present in an amount of about 30 LF and said tetanus toxoid is present in an amount of about 10 LF.

11. The vaccine of claim 3 wherein said inactivated polio virus comprises a mixture of inactivated polio virus types 1, 2 and 3.

12. The vaccine of claim 11 wherein said inactivated polio virus comprises a mixture of inactivated poliovirus types 1, 2 and 3 in the proportions:
about 20 to about 50 D antigen units of poliovirus type 1;
about 4 to about 10 D antigen units of poliovirus type 2; and
about 8 to about 40 D antigen units of poliovirus type 3 in a single human dose.

13. The vaccine of claim 12, wherein said inactivated poliovirus comprises a mixture of inactivated poliovirus types 1, 2 and 3 in the proportions:
about 40 D antigen units of poliovirus type 1;
about 8 D antigen units of poliovirus type 2; and
about 32 D antigen units of poliovirus type 3 in a single human dose.

14. The vaccine of claim 3 wherein said conjugate comprises a conjugate of tetanus toxoid or diphtheria toxoid and polyribose ribitol phosphate (PRP) of *Haemophilus influenzae* type b.

15. The vaccine of claim 3 wherein the Hepatitis B surface antigen is separated from other components in a dual-chamber syringe and is reconstituted during the administration to the subject

16. A multi-valent vaccine composition comprising, per 0.5 ml dose,
25 ug pertussis toxoid;
25 ug filamentous hemagluttinin;
30 LF diphtheria toxoid;
10 LF tetanus toxoid;
40 D antigen units poliovirus type 1;

8 D antigen units poliovirus type 2;
32 D antigen units poliovirus type 3;
10 ug Haemophilus influenzae type b polysaccharide covalently bound to 20 ug tetanus toxoid;
5 ug Hepatitis B Surface Ag;
20 µMoles phosphates
5 µMoles carbonates
0.125 ml tris 50mMolaire buffer comprising saccharose in 42,5 %
and 0.306 mg aluminum hydroxide.

17. A method of immunizing a human host against disease caused by infection by *Bordetella pertussis, Clostridium tetanae, Corynebacterium diphtheriae, Haemophilus influenzae, poliovirus and/or Hepatitis b virus, which method comprises administering to the host an immunoeffective dose of the immunogenic composition of claim 1.*

18. The method of claim 17, wherein the host is a child.

19. A multivalent vaccine of claim 16 wherein the aluminium is in a quantity of 0.356 mg and wherein the Hepatitis B Surface Ag is separated from other components in a multi-chamber syringe.

20. A multivalent vaccine of claim 16 wherein the Hepatitis B Surface Ag is adsorbed on aluminium salts.

Case No.: MBHB00-210

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

MULTIVALENT VACCINES

the specification of which is attached hereto unless the following space is checked:

☒ was filed on March 13, 2000 as United States Application Serial Number 09/508,570.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT international application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s):

	Number	Country	Day/Month/Year Filed
1.	PCT/EP97/05378	PCT	15 September 1997
2.			

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below:

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I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or § 365(c) of any PCT international application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R. § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

	Application Number	Filing Date	Status: patented, pending, abandoned
1.			

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In addition, the undersigned appoints John E. Parrish (Reg. No. 35,315), Timothy Howe (Reg. No. 39,228), and G. Kenneth Smith (Reg. No. 43,135) as our agents.

The mailing address and telephone number of each of whom is **CONNAUGHT LABORATORIES, INC.**, Route 611, P.O. Box 187, Swiftwater, Pennsylvania 18370-0187, United States of America, and (717) 839-5027, with full power of substitution and revocation to prosecute this application and to transact all business in the United States Patent and Trademark Office connected therewith, throughout the world with respect thereto for all corresponding applications, and to represent the undersigned before all the competent International Authorities in connection with the above identified application filed with the USPTO, and to make and receive payments on behalf of the undersigned.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Full name of third joint inventor: Sandrine Lentsch-Graf

Inventor's signature: _____ Date: _____
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Full name of fourth joint inventor: Laurent Marchal

Inventor's signature: L. Marchal Date: 04.10.00
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Case No.: MBHB00-210

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Application Number	Filing Date	Status: patented, pending, abandoned

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Inventor's signature: _____ Date: March 22nd, 2000

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